FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **21133US0XPCT**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): 09/926674

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/PT00/00004	**31 May 2000**	**31 May 1999**

TITLE OF INVENTION
CULTURE MEDIUM FOR THE DETECTION OF ZYGOSACCHAROMYCES

APPLICANT(S) FOR DO/EO/US
Cecilia LEAO, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

PC/IB/304 Amended Sheets (Pages 25 and 26)
PCT/IB/308
Notice of Priority
Form PTO 1449

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/926677	PCT/PT00/00004	21133US0XPCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1040.00	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$890.00	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$740.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$710.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$890.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =	0	x $18.00	$0.00
Independent claims	- 3 =	0	x $84.00	$0.00
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00

TOTAL OF ABOVE CALCULATIONS =	$1,020.00
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	$0.00
SUBTOTAL =	$1,020.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$0.00
TOTAL NATIONAL FEE =	$1,020.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	$0.00
TOTAL FEES ENCLOSED =	$1,020.00
Amount to be: refunded	$
charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Norman F. Oblon
NAME

24,618
REGISTRATION NUMBER

Nov. 30 2001
DATE

211333US-0XPCT

#3

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

CECILIA LEAO ET AL : ATTN: APPLICATION DIVISION

SERIAL NO: 09/926,677 :

FILED: NOVEMBER 30, 2001 :

FOR: CULTURE MEDIUM FOR THE DETECTION OF ZYGOSACCHAROMYCES :

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please replace the paragraph beginning on page 1, line 26 through page 2, line 6 with the following paragraph.

Heretofore, the study of the yeast microflora present in the most diverse habitats (e.g. food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a large range of physiological and biochemical tests. It is a demanding work that

only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.

Please replace the paragraph beginning on page 7, line 1 through page 7, line 7 with the following paragraph.

Figure 5 shows the morphology of *P. membranifaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.

Please replace Table 2 on page 8 with the following Table.

Table 2 Oligoelements and vitamin solutions composition

Compound			Concentration (%)	
Oligoelements Solution A	Boric Acid	H_3BO_3	0.1	(w/v)
	Potassium Iodide	KI	0.02	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.04	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.008	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	0.04	(w/v)
	Manganese sulphate tetrahydrate	$MnSO_4 \cdot 4H_2O$	0.08	(w/v)
	Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.08	(w/v)
	Hydrochloric acid	HCl 10^{-3}N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2$ Ca	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_6H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

--

Please replace the paragraph on page 9, lines 1-3 with the following paragraph.

The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the sterilization is accomplished in autoclave at 121 °C, for 20 minutes. The pH must be adjusted to 4.5.

Please replace the paragraph beginning on page 9, lines 5-11 with the following paragraph.

The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with NaOH 10M. The sterilization is accomplished by filtration. This solution and the base are brought to 50±5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.

Please replace Table 3 with the following Table.

Table 3 Inoculation by streaking - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours

Please replace Table 4 on page 11 with the following Table.

Table 4 Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

*the change in the medium color was observed after an additional incubation period of 72-96 hours

Please replace Table 5 on page 13 with the following Table.

Table 5 Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours

Please replace the paragraph beginning on page 13, line 14 through page 14, line 8 with the following paragraph.

A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 μm), the filters are then placed on Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (medium containing yeast extract, peptone, and glucose).

Please replace the paragraph beginning on page 14, line 10 through page 14, line 14 with the following paragraph.

The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P. membranifaciens* and *D. anomala* was significantly reduced, lower the 0.01%.

Please replace the paragraph beginning on page 14, line 16 through line 19 with the following paragraph.

S. cerevisiae, P. membranifaciens and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.

Please replace Table 6 with the following Table.

Table 6 Recovery ration (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii *Saccharomyces cerevisiae*	57 n.d.
Zygosaccharomyces bailii *Pichia membranifaciens* *Dekkera anomala*	67 n.d. n.d.
Saccharomyces cerevisiae	< 0,002
Pichia membranifaciens	0,011
Dekkera anomala	< 0,004

n.d. not determined

Please replace the paragraph on page 15a, lines 7-11 with the following paragraph.

In mixed cultures of *P. membranifaciens* and *Z. bailii*, an intense blue coloring of the colonies formed by *P. membranifaciens* was observed. This characteristic is equally due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. However, the discrimination between those colonies is clear as can be seen in the appended Figure 5.

Please replace Table 8 on page 17 with the following Table.

Table 8 Application of cell suspensions drops on the surface of solid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranifaciens	13	green	green
Debaryomyces hansenii	2	green	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

Please replace Table 9 on page 18 with the following Table.

Table 9 Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranifaciens	13	green	green
Debaryomyces hansenii	2	green	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

Please replace the paragraph beginning on page 18, line 22 through page 19, line 2 with the following paragraph.

As in Example 6 the results obtained show that, for 0,3% acid formic concentration, the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, inoculated in liquid media after a minimum incubation period of 48 hours. The same is valid for the detection of *Z. bailii* in a medium with 0,5% formic acid concentration.

Please replace Table 10 on page 19 with the following Table.

Table 10 Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii *Saccharomyces cerevisiae*	82 n.d.	81 n.d.	35 n.d.
Zygosaccharomyces bailii *Pichia membranifaciens* *Dekkera anomala*	99 n.d. n.d.	94 n.d. n.d.	34 n.d. n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia membronifaciens	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined

IN THE CLAIMS

Please amend the claims as shown in the marked-up copy following this amendment to read as follows.

1. (Amended) A differential and selective culture medium for *Zygosaccharomyces ballii* and *Zygosaccharomyces bisporus* yeasts, wherein said medium comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator having a pKi between 4.5 and 4.8 and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. (Amended) Culture medium according to claim 1 wherein glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. (Amended) Culture medium according to claim 1 wherein formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. (Amended) Culture medium according to claim 3 wherein the formic acid concentration is preferably 0.4% (v/v).

5. (Amended) Culture medium according to claim 1 wherein the base mineral medium comprises ammonium sulphate (0.5% (w/v)), potassium dihydrogenphosphate (0.5% (w/v)), magnesium sulphate heptahydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (0.1% (w/v)), potassium iodide (0.02% (w/v)) and sodium molibdate dihydrate (0.04% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate (0.008% (w/v)), iron chloride hexahydrate (0.04% (w/v)), manganese sulphate tetrahydrate (0.08% (w/v)), zinc sulphate heptahydrate (0.08% (w/v)) and hydrochloric acid (HCL 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001 % (w/v)), calcium panthotenate (0.08% (w/v)), mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. (Amended) Culture medium according to claim 1 wherein the acid-base indicator is one having a pKi between 4.5 and 4.8, preferably bromocresol green.

7. (Amended) Culture medium according to claim 6 wherein the pH is adjusted to 4.3-4.8, preferably 4.5.

8. (Amended) Culture medium according to claim 1 wherein said medium further contains an antibiotic inhibitor of bacterial growth, in the usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. (Amended) A culture medium according to claim 1, wherein said medium contains all the ingredients except agar, that is in its liquid form.

10. (Amended) A differential and selective culture medium for *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, wherein said medium is composed of

Glucose	0.1 % (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogenosulphate	0.5% (w/v)
Magnesium sulphate hetpahydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	0.1% (w/v)
Potassium Iodide	0.02% (w/v)
Sodium molibdate dihydrate	0.04% (w/v)
Oligoelements Solution B	0.05% (v/v)
Copper sulphate pentahydrate	0.008% (w/v)
Iron chloride hexahydrate	0.04% (w/v)
Manganese sulphate tetrahydrate	0.08% (w/v)
Zinc sulphate heptahydrate	0.08% (w/v)
Hydrochloric acid, HCI 10^{-3}N,	0.8% (v/v)
Vitamin Solution	0.05% (v/v)

Biotin	0.001 % (w/v)
Calcium panthotenate	0.08% (w/v)
Mioinositol	4.0% (w/v)
Niacin	0.16% (w/v)
Pyridoxine hydrochloride	0.16% (w/v)
Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH 4.5 with NaOH 10M.

11. (Amended) Culture medium according to claim 1, wherein the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, bringing this solution and the base medium at about 50 ±5°C, before mixing the same and adjusting the final pH value to the desired value.

12. (Amended) Process for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts comprising the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator having a pKi between 4.5 and 4.8 and, optionally an antibiotic inhibitor of bacterial growth and agar.

14. (Amended) Process according to claim 12, wherein said process is applied to the detection and numbering yeasts of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* species in wines, as well as in other beverages or food containing or not mixed yeast populations.

15. (Amended) Use of a culture medium according to claim 1, to be included in yeast identification galleries.

16. (Amended) Use of a culture medium according to claim 1 in an industry, particularly in the quality and process control in the food and beverage industry.

REMARKS

The specification has been amended to correct clerical errors. The claims have been amended to correct clerical errors, for clarity, and to remove multiple dependencies. No new matter is believed to have been added. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

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Marked-Up Copy
Serial No:

Amendment Filed on:
3-11-2002

IN THE SPECIFICATION

Please replace the paragraph beginning on page 1, line 26 through page 2, line 6 with the following paragraph.

--Heretofore, the study of the yeast microflora present in the most diverse habitats (e.g. food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a large range of [physiologic] physiological and biochemical tests. It is a demanding work that only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.--

Please replace the paragraph beginning on page 7, line 1 through page 7, line 7 with the following paragraph.

--Figure 5 shows the morphology of *P.* [*membranaefaciens*] *membranifaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the

use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.--

Please replace Table 2 on page 8 with the following Table.

--Table 2 Oligoelements and vitamin solutions composition

Compound			Concentration (%)	
Oligoelements Solution A	Boric Acid	H_3BO_3	[1.0] 0.1	(w/v)
	Potassium Iodide	KI	[0.2] 0.02	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	[0.4] 0.04	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	[0.08] 0.008	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	[0.4] 0.04	(w/v)
	Manganese sulphate tetrahydrate	$MnSO_4 \cdot 4H_2O$	[0.8) 0.08	(w/v)
	Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	[0.8] 0.08	(w/v)
	Hydrochloric acid	HCl 10^{-3}N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2$ Ca	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_6H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

--

Please replace the paragraph on page 9, lines 1-3 with the following paragraph.

--The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the sterilization is accomplished in autoclave at 121 °C, for 20 minutes. The pH must be adjusted to 4.5.--

Please replace the paragraph beginning on page 9, lines 5-11 with the following paragraph.

--The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with [HCl 1M] NaOH 1OM. The sterilization is accomplished by filtration. This solution and the base are [mixed] brought to 50±5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.--

Please replace Table 3 with the following Table.

--Table 3 Inoculation by streaking - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia [membranaefaciens] membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours--

Please replace Table 4 on page 11 with the following Table.

--Table 4 Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia [membranaefaciens] membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

*the change in the medium color was observed after an additional incubation period of 72-96 hours--

Please replace Table 5 on page 13 with the following Table.

--Table 5 Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces bisporus	3	blue*
Zygosaccharomyces nouxii	6	green
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia [membranaefaciens] membranifaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours--

Please replace the paragraph beginning on page 13, line 14 through page 14, line 8 with the following paragraph.

--A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 μm), the filters are then placed on Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (medium containing yeast extract, peptone, and glucose).--

Please replace the paragraph beginning on page 14, line 10 through page 14, line 14 with the following paragraph.

--The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P.* [*membranaefaciens*] *membranifaciens* and *D. anomala* was significantly reduced, lower the 0.01%.--

Please replace the paragraph beginning on page 14, line 16 through line 19 with the following paragraph.

--*S. cerevisiae, P.* [*membranaefaciens*] *membranifaciens* and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.--

Please replace Table 6 with the following Table.

--Table 6 Recovery ration (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii *Saccharomyces cerevisiae*	57 n.d.
Zygosaccharomyces bailii *Pichia [membranaefaciens] membranifaciens* *Dekkera anomala*	67 n.d. n.d.
Saccharomyces cerevisiae	< 0,002
Pichia [membranaefaciens] membranifaciens	0,011
Dekkera anomala	< 0,004

n.d. not determined--

Please replace the paragraph on page 15a, lines 7-11 with the following paragraph.

--In mixed cultures of *P.* [*membranaefaciens*] *membranifaciens* and *Z. bailii*, an intense blue coloring of the colonies formed by *P.* [*membranaefaciens*] *membranifaciens* was observed. This characteristic is equally due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. However, the discrimination between those colonies is clear as can be seen in the appended Figure 5.--

Please replace Table 8 on page 17 with the following Table.

--Table 8 Application of cell suspensions drops on the surface of solid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia [membranaefaciens] <u>*membronifaciens*</u>	13	green	green
Debaryomyces hansenii	2	green	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined--

Please replace Table 9 on page 18 with the following Table.

--Table 9 Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia [membranaefaciens] membranifaciens	13	green	green
Debaryomyces hansenii	2	green	green

*the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined--

Please replace the paragraph beginning on page 18, line 22 through page 19, line 2 with the following paragraph.

--As in Example 6 the results obtained show that, for 0,3% acid formic concentration, the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, inoculated in liquid media after a minimum incubation period of 48 hours. The same is valid for the detection of *Z. bailii* in a medium with 0,5% [acid formic] formic acid concentration.--

Please replace Table 10 on page 19 with the following Table.

--Table 10 Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii *Saccharomyces cerevisiae*	82 n.d.	81 n.d.	35 n.d.
Zygosaccharomyces bailii *Pichia [membranaefaciens]* *membranifaciens* *Dekkera anomala*	99 n.d. n.d.	94 n.d. n.d.	34 n.d. n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia [membranaefaciens] *membranifaciens*	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined--

IN THE CLAIMS

Please amend the claims to read as follows.

--1. (Amended) A differential and selective culture medium for *Zygosaccharomyces ballii* [e] and *Zygosaccharomyces bisporus* yeasts, [characterized in that it] wherein said medium comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator having a pKi between 4.5 and 4.8 and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. (Amended) Culture medium according to claim 1 [characterized in that] wherein glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. (Amended) Culture medium according to claim 1 [characterized in that] wherein formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. (Amended) Culture medium according to claim 3 [characterized in that] wherein the formic acid concentration is preferably 0.4% (v/v).

5. (Amended) Culture medium according to claim 1 [characterized in that] wherein the base mineral medium comprises ammonium sulphate (0.5% (w/v)), potassium dihydrogenphosphate (0.5% (w/v)), magnesium sulphate heptahydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid ([1.0%] 0.1% (w/v)), potassium iodide ([0.2%] 0.02% (w/v)) and sodium molibdate dehydrate ([0.4%] 0.04% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate ([0.08%] 0.008% (w/v)), iron chloride hexahydrate ([0.4%] 0.04% (w/v)), manganese sulphate tetrahydrate ([0.8%] 0.08% (w/v)), zinc sulphate heptahydrate ([0.8%] 0.08% (w/v)) and hydrochloric acid (HCL 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001 % (w/v)), calcium panthotenate (0.08% (w/v)), mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. (Amended) Culture medium according to claim 1 [characterized in that] wherein the acid-base indicator is one having a pKi between 4.5 and 4.8, preferably bromocresol green.

7. (Amended) Culture medium according to claim 6 [characterized in that] wherein the pH is adjusted to 4.3-4.8, preferably 4.5.

8. (Amended) Culture medium according to claim 1 [characterized in that it] wherein said medium further contains an antibiotic inhibitor of bacterial growth, in the

usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. (Amended) A culture medium according to [any previously claim characterized in that it] claim 1, wherein said medium contains all the ingredients except agar, that is in its liquid form.

10. (Amended) A differential and selective culture medium for *Zygosaccharomyces bailii* [e] and *Zygosaccharomyces bisporus* yeasts, [characterized in that it] wherein said medium is composed of

Glucose	0.1 % (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogenosulphate	0.5% (w/v)
Magnesium sulphate hetpahydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	[1.0%] 0.1% (w/v)
Potassium Iodide	[0.2%] 0.02% (w/v)
Sodium molibdate dihydrate	[0.4%] 0.04% (w/v)
Oligoelements Solution B	0.05% (v/v)
Copper sulphate pentahydrate	[0.08%] 0.008% (w/v)
Iron chloride hexahydrate	[0.4%] 0.04% (w/v)

Manganese sulphate tetrahydrate	[0.8%] 0.08% (w/v)
Zinc sulphate heptahydrate	[0.8%] 0.08% (w/v)
Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)
Vitamin Solution	0.05% (v/v)
Biotin	0.001 % (w/v)
Calcium panthotenate	0.08% (w/v)
Mioinositol	4.0% (w/v)
Niacin	0.16% (w/v)
Pyridoxine hydrochloride	0.16% (w/v)
Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH [4.6] 4.5 with [HCl 1M] NaOH 10M.

11. (Amended) Culture medium according to [any previously claim characterized in that] claim 1, wherein the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, [annealing] bringing this solution and the base medium at about 50 ±5°C, before mixing the same and [to adjust] adjusting the final pH value to the desired value.

12. (Amended) Process for the detection of *Zygosaccharomyces bailii* [e] and *Zygosaccharomyces bisporus* yeasts [characterized by] comprising the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only

carbon and energy sources, an appropriated acid-base indicator having a pKi between 4.5 and 4.8 and, optionally an antibiotic inhibitor of bacterial growth and agar.

14. (Amended) Process according to [claims 12 and 13 characterized in that it] claim 12, wherein said process is applied to the detection and numbering yeasts of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* species in wines, as well as in other beverages or food containing or not mixed yeast populations.

15. (Amended) Use of a culture medium according to [claims 1 to 11] claim 1, to be included in yeast identification galleries.

16. (Amended) Use of a culture medium according to [claims 1 to 11] claim 1 in an industry, particularly in the quality and process control in the food and beverage industry.--

Culture medium for ~~the~~ detection of *Zygosaccharomyces ~~bailii and Zygosaccharomyces bisporus~~* Yeasts~~/~~

Object of the Invention

The present invention refers to a differential and selective culture medium containing glucose, formic acid and an acid-base indicator, for the detection in a sample, after 48 hours, of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, two of the most dangerous species when considering food deterioration, and to a process for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts using the referred culture medium. ~~It is~~ It is a further object of the present invention the use of the referred culture medium in a gallery of yeasts identification tests.

State of the prior art

Yeasts are a growing problem in the food industry. The use of milder preservation processes in order to maintain the organoleptic properties of the product, of packages with modified atmospheres, and of new formulations, designed to avoid bacterial contamination are, nevertheless, favorable to yeast contamination. Although some pathogenic yeast species have been detected in food and the opportunistic strains may be dangerous to a fraction of the population, the fundamental risk of contamination that arises is not one of sanitary nature, but it consists in the spoilage effects that certain yeasts, such as *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* have in food products, with the consequent economic losses involved.

Heretofore, the study of the yeast microflora present in the most diverse habitats (*e.g.* food, nature), comprises a first strain isolation stage, using the general selective yeast culture media, and a second identification stage of the isolated strains, through the use of conventional and/or molecular biology based methods. The classical yeast identification methods are based in a series of vegetative and sexual reproduction characteristics, and comprise a

large range of physiologic and biochemical tests. It is a demanding work that only produces results after at least one to two weeks, and requires a great deal of experience for the correct interpretation of the results. The molecular biology based methods are, generally, faster than the classical ones, but they also require a good amount of operator experience and involve expensive equipment and reactants.

There are some culture media commercially available for the detection of yeasts in wines, namely the Wallerstein Laboratory Nutrient Medium, WLN, used for detecting fermenting yeasts, and the Wallerstein Laboratory Differential Medium, WLD, which allow the detection of lactic and acetic bacteria as well as of yeasts belonging to the non-fermenting flora (both from Difco). However, these prior art media are not capable to differentiate the yeasts, particularly the *Zygosaccharomyces bailii* species.

There is therefore the necessity for a culture medium and a process for the detection and identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, rapid and efficient, and which is thus an alternative means to the conventional techniques for the rapid detection of these species.

Description of the invention

It was surprisingly found that *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, when grown in a medium containing glucose, formic acid and an appropriated acid-base indicator, lead to a rapid change in the medium color and to the formation of colored colonies after at least 48 hours, these changes being characteristic and exclusive of the referred yeasts in the referred culture medium.

It was also found that the medium according to the invention is differential for the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts,

through the inclusion of an appropriate acid-base indicator, and can be selective for the growth of the referred yeasts, depending on the formic acid concentration present in the medium.

Thus, according to the present invention, a new differential and selective culture medium was developed, which permits the identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts, assuring results after 48 hours of incubation, and which is therefore an alternative means to the conventional techniques for the rapid detection of these species, allowing a drastic reduction in the time and work involved in their identification.

The culture medium according to the present invention comprises a base mineral medium, supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator, namely one having a pK_i between 4.5 and 4.8, particularly bromocresol green, and optionally agar and an antibiotic inhibitor of bacterial growth, such as cloramphenicol.

According to the present invention, the formic acid is present in the culture medium in a concentration from 0.1% to 0.5% (v/v), the concentration being selected depending if the culture medium is to be selective or only differential.

When the concentration of formic acid is increased in the culture medium according to the present invention, the selectivity of the medium for *Z. bailii* and *Z. bisporus* yeasts increases, although at expenses of some differentiability, as shown in examples 6 and 7 below.

According to the present invention, the glucose is present in a concentration from 0.05% and 0.1% (p/v), preferably 0,1% (p/v).

The culture medium according to the present invention further allows, through the choice of appropriated conditions, in particular the inoculation methodology, the enumeration of *Z. bailii* and *Z. bisporus* yeasts in a sample, regardless the presence of other yeasts, since it is selective as shown in examples 4 and 8.

In an embodiment of the present invention, the acid-base indicator is bromocresol green which provides the medium with a green color, that is converted to blue by the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts. Additionally, the colonies of the referred yeasts present, in the medium of the invention, a blue coloring.

In another embodiment, the culture medium according to the invention may contain additionally an inhibitor of bacterial growth, being particularly useful for application in samples of mixed populations including bacteria.

The culture medium object of the invention is prepared by autoclave sterilization of the base mineral medium in deionized water. The medium is then allowed to cool, and before solidifying, the glucose, formic acid, oligoelements and vitamins, prepared as adequate solutions and previously sterilized, are added under aseptic conditions. The whole medium is homogenized and aseptically dispensed into Petri dishes.

The present invention also refers to a process of detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts present in a sample, using a culture medium according to the present invention, as characterized above.

According to this feature of the present invention, a process was developed which comprises: (i) preparing a medium according to the present invention; (ii) inoculating it, by spreading, streaking or by deposition of a drop of cell

suspension, with a sample to be analyzed for *Zygosaccharomyces bailii* and/or *Zygosaccharomyces bisporus* yeasts; (iii) incubating it in a incubator at a suitable temperature and for a time enough for the yeast development (minimum 48 hours); and (iv) observing the color changing in the medium and the colonies formation, to conclude the presence of the referred yeasts when occurs a changing of the medium color and formation of colored colonies in agreement with the acid-base indicator used.

The present invention can be used with previously isolated and purified strains, there being no kind of limitation concerning the type of inoculation that is used. However, the time needed to observe the turning of the indicator depends on the cell concentration of the inoculum and on the method of inoculation.

The present invention can also be used with cell suspensions of mixed yeast populations, containing yeasts other than *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*, providing information about the presence of these species, every time that blue colonies are detected in conjunction with a change in the medium color.

One of the objects of the present invention is to provide the food industry, particularly the wine and beverages industry, with a procedure for the detection of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts. The procedure is simple and easily reproducible by any microbiological analysis laboratory. Additionally, the production of the culture medium doesn't require new technologies. Once prepared, the culture medium finds immediate use in any industrial facility or quality control laboratory, since there is no need for highly skilled personnel other than the one in charge of the routine microbiological analyses.

Further, the culture medium according to the present invention can be used to integrate galleries of identification of yeasts.

Brief Description of the Figures

Figure 1 is a photograph showing the response of several yeasts (*Z. bailii* ISA 1265 and *Z. bailii* IGC 4806 ~~3806~~: positive response; *T. delbrueckii* ISA 1229 and *I. Orientalis* IGC 3806: negative response) in a solid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% v/v) at the end of 96 hours of incubation at 30°C. The *Z. bailii* yeasts shown a positive response revealed by a blue coloring of the culture medium in the dish, while the negative responses are shown as a green coloring which did not change during the incubation.

Figure 2 is a photograph showing the response of several yeasts in a liquid medium according to the present invention containing glucose (0.1% w/v) and formic acid (0.3% w/v) at the end of 48 hours of incubation at 30°C. All the *Z. bailii* strains induced the medium to change color to blue, while all the others maintained the green color.

Figure 3 shows the morphology of *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.3% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The colonies can be observed well defined with a blue color.

Figure 4 shows the morphology of *S. cerevisiae* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies shown a blue coloring, perfectly distinct from the creme coloring of the other colonies.

Figure 5 shows the morphology of *P. membranaefaciens* and *Zygosaccharomyces bailii* yeast colonies in a culture medium according to the present invention containing 0.2% (v/v) of formic acid and 0.1% (w/v) of glucose, obtained by the use of the method of membrane filtration, after 96 hours of incubation at the temperature of 30°C. The *Z. bailii* colonies are totally distinguishable by its morphology and blue color.

Preferred embodiments of the invention

In a preferred embodiment of the present invention the differential and selective culture medium, for identification of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts in a sample, after at least 48 hours of incubation, comprises a base mineral medium, including bromocresol green as the acid-base indicator, supplemented with oligoelements and vitamins, 0.05% to 0.1% (w/v) of glucose and 0.1% to 0.5% (v/v) of formic acid as the only energy and carbon sources, and optionally agar and an inhibitor of bacterial growth.

In this embodiment of the invention, the bromocresol green provides the medium with a green coloring that will be converted into blue through the action of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts during incubation under appropriate conditions. Additionally, the colonies of these yeasts will also present a blue color. The change of color of the culture medium is characteristic of these yeast species, as illustrated in examples 1 and 2, thus allowing the detection of the presence thereof in a sample only by the color changing.

The process according to the present invention will now, be illustrated by means of the non limitative examples below:

Examples

Example 1

This example illustrates the preparation of a solid culture medium according to the present invention and shows that it is effective in the identification of *Z. bailii* and *Z. bisporus* yeasts.

A culture medium is prepared comprising the following ingredients:

Table 1 **Culture medium composition for the detection of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts**

Compound			Concentration (%)	
Base Medium	Ammonium sulphate	$(NH_4)_2SO_4$	0.5	(w/v)
	Potassium dihydrogen phosphate	KH_2PO_4	0.5	(w/v)
	Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	0.05	(w/v)
	Calcium chloride dihydrate	$CaCl_2 \cdot 2\ H_2O$	0.013	(w/v)
	Bromocresol green	$C_{21}H_{14}Br_4O_5S$	0.005	(w/v)
	Agar	-	2.0	(w/v)
Glucose	-	$C_6H_{12}O_6$	0.1	(w/v)
Formic acid	-	CH_2O_2	0.4	(v/v)
Oligoelements Solution A	(Composition according to Table 2)	-	0.05	(v/v)
Oligoelements Solution B	(Composition according to Table 2)	-	0.05	(v/v)
Vitamin Solution	(Composition according to Table 2)	-	0.05	(v/v)

Table 2 **Oligoelements and vitamin solutions composition**

Compound			Concentration (%)	
Oligoelements Solution A	Boric acid	H_3BO_3	1.0	(w/v)
	Potassium Iodide	KI	0.2	(w/v)
	Sodium molibdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.4	(w/v)
Oligoelements Solution B	Copper sulphate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.08	(w/v)
	Iron chloride hexahydrate	$FeCl_3 \cdot 6\ H_2O$	0.4	(w/v)
	Manganese sulphate hexahydrate	$MnSO_4 \cdot 4H_2O$	0.8	(w/v)
	Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	0.8	(w/v)
	Hydrochloric acid	HCl 10 ^{3}N	0.8	(v/v)
Vitamin Solution	Biotin	$C_{10}H_{16}N_2O_3S$	0.001	(w/v)
	Calcium panthotenate	$C_9H_{16}NO_5 \cdot 1/2\ Ca$	0.08	(w/v)
	Mioinositol	$C_6H_{12}O_6$	4.0	(w/v)
	Niacin	$C_6H_5NO_2$	0.16	(w/v)
	Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	0.16	(w/v)
	Thiamin hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	0.16	(w/v)

The base medium compounds are dissolved in 4/5 of the estimated deionized water volume, and the sterilization is accomplished in autoclave at 121°C, for 20 minutes.

The other medium compounds (glucose, formic acid, oligoelements solution A, oligoelements solution B, and vitamin solution) are dissolved in the remaining water volume so that the final concentration of these compounds equals the values mentioned in Table 1. The pH must be adjusted to 4.5 with HCl 1M. The sterilization is accomplished by filtration. This solution and the base medium are ~~annealed~~ mixed at 50±5°C before being mixed together. The whole medium is homogenized and dispensed into Petri dishes.

The yeast strains to be identified, previously purified and inoculated in agar slants with a generic yeast culture medium (yeast extract medium, peptone, and glucose), are incubated for 48 hours at 28°C. An loopful is transferred to the culture medium with glucose and formic acid, prepared above. The inoculation is made by streaking and the plates are incubated at 30°C, for a minimum time of 48 hours. Alternatively, the inoculation may be done with a cotton smear containing an equivalent biomass amount.

The results obtained are presented in Table 3. Typical responses of the below mentioned yeasts are shown in Figure 1.

Table 3 **Inoculation by streaking - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~fouxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Toruiaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 24-48 hours

A change in the culture medium color from green to blue was observed in all of the tested *Z. bailii* strains. However, it was observed that 3 of the 8 tested *Z. bisporus* strains converted the medium color only after an additional incubation period of 24 to 48 hours. For all the strains of the other species tested a negative result was observed, since the medium color did not change.

The results that were obtained show that the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* directly inoculated from cultures in solid medium after a minimum incubation period of 48 hours.

(*) Zygosaccharomyces rouxii 6 green

Example 2

The same procedure as Example 1 was used, differing only in that the inoculation was made with single strain cell suspensions instead of cells originated in solid medium. The cells are also originated from agar slants as disclosed in Example 1. The cell suspensions are prepared in deionized water in such a way that the optical density (OD_{640}) lies within the range of 0.7 to 1.0. 10 µl drops of these suspensions are placed on the surface of Petri dishes containing the medium disclosed in Example1. The plates were incubated at 30°C for 48 hours.

The results obtained are presented in Table 4. These results are ~~similar~~ identical to the ones presented for Example 1.

Table 4 **Application of cell suspensions on the surface of the solid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~fouxii~~ bisporus (*)	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Ledderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 72-96 hours

(*) (see page 10)

The culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 3

The same procedure as Example 2 was used, but using the culture medium in its liquid form. 25 µl of the cell suspension are transferred to 225 µl of the medium disclosed in Example 1 but without the agar (contained in the wells of a microplate), and in a concentration such that after the 25 µl addition of the cell suspension the final medium components concentration equals the ones disclosed in Example 1. The incubation conditions are similar to those described in Example 2. In addition the culture is homogenized by mechanical mixing at 160 rpm.

The results obtained are presented in Table 5. These results are similar to the ones obtained in the above examples.

Table 5 **Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid (0.4% v/v) after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color
Zygosaccharomyces bailii	15	blue
Zygosaccharomyces bisporus	5	blue
Zygosaccharomyces ~~louxii~~ bisporus	3	blue*
Zygosaccharomyces florentinus	1	green
Saccharomyces bayanus	2	green
Saccharomyces cerevisiae	21	green
Saccharomyces pastorianus	2	green
Saccharomycodes ludwigii	3	green
Schizosaccharomyces pombe	4	green
Pichia membranaefaciens	13	green
Pichia anomala	7	green
Dekkera anomala	3	green
Dekkera bruxellensis	4	green
Debaryomyces hansenii	2	green
Issatchenkia orientalis	6	green
Kluyveromyces marxianus	5	green
Kloeckera apiculata	1	green
Lodderomyces elongisporus	2	green
Rhodotorula mucilaginosa	2	green
Torulaspora delbrueckii	7	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

The culture medium according to the present invention, in the liquid form, is equally suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions after a minimum incubation period of 48 hours.

Example 4

This Example shows that the culture medium according to the present invention is selective for yeasts of the *Z. bailii* and *Z. bisporus* species in samples of mixed yeasts populations.

A similar procedure as in Example 3 is used, differing only in that the cell suspensions used are pure or mixed (in equal ratios) yeast cell suspensions, and in that the method of membrane filtration is used. The cell suspension is prepared as in Example 2. The mixed cultures are prepared from pure culture

(See page 10)

suspensions. In this case, the inoculations are accomplished using an aliquot of the suitably diluted suspension that is filtered under vacuum through a sterilized filtration membrane (pores of 0.45 μm), the filters are then placed Petri dishes, and the dishes containing the filters on the surface of the medium disclosed in Example 1, are incubated at 30°C for 96 hours. As a reference culture medium (corresponding to a recovery ratio of 100%) a generic yeast culture medium is used (yeast extract ~~medium~~, peptone, and glucose). medium containing

The results obtained are presented in Table 6. The recovery ratio of *Z. bailii* cells in the medium disclosed in Example 1 is about 60 to 70 %, regardless of the presence of other yeast species. The culture medium was shown to be highly selective since the recovery ratio of *S. cerevisiae, P. membranaefaciens* and *D. anomala* was significantly reduced, lower the 0.01%.

S. cerevisiae, P. membranaefaciens and *D. anomala* being representative examples of contaminant species in wines, the culture medium according to the invention will be useful and appropriate for the identification of *Z. bailii* in contaminated wines samples.

Table 6 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio
Zygosaccharomyces bailii	65
Zygosaccharomyces bailii	57
Saccharomyces cerevisiae	n.d
Zygosaccharomyces bailii	67
Pichia membranaefaciens	n.d.
Dekkera anomala	n.d
Saccharomyces cerevisiae	< 0,002
Pichia membranaefaciens	0,011
Dekkera anomala	<0,004

n.d. not determined

The Figures 3, 4, and 5 show the colony morphology of different species in pure and mixed cultures, being remarkable the easy discrimination between the 3 species using only the colonies color and morphology.

~~In some cases non typical colonies (ca. 2-3%) with a light blue coloring or with an intense blue coloring can be present. The first of these (Fig. 4), with a morphology similar to that of *S. cerevisiae*, were judged as belonging to this species. The light coloring of these colonies is due to the incorporation of the indicator after the color change induced by the presence of *Z. bailii*. The second kind of colonies (Fig. 5), with a similar morphology to that of *P. membranaefaciens* were judged as belonging to this species, the intense coloring being due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii*. This characteristic was equally observed for the pure cultures of *P. membranaefaciens* that showed a very intense green coloring in contrast with those of *S. cerevisiae*, that under these conditions, showed a green cream coloring. However, the discrimination between these colonies is clear as can be seen in the appended Figures 4 and 5.~~ (insert page 15a)

Example 5

This example shows the differential ability of the culture medium according to the present invention and the enumeration of *Z. bailii* cells in wine samples.

The enumeration of *Z. bailii* cells in wine samples is made using membrane filtration (according to the method disclosed in Example 4). For the determination of the number of colony forming units (CFU)/ml of wine is done after 96 hours of incubation at the temperature of 30°C. Other commercial culture media presently used for the detection of yeasts in wines (Wallerstein Laboratory Differential Medium, WLD, and Wallerstein Laboratory Nutrient Medium, WLN, both marketed by Difco) are tested in parallel. The WLN medium is used for the detection of fermenting yeasts, while the WLD

(new page)

In mixed cultures of *S. cerevisiae* and *Z. bailii*, a few colonies (*ca.* 2-3%) with a light blue coloring and with a morphology similar to that of *S. cerevisiae* can be present, and that were judged as belonging to this species. The light coloring is due to the affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii.*

In mixed cultures of *P. membranaefaciens* and *Z. bailii,* an intense blue coloring of the colonies formed by *P. membranaefaciens* was observed. This characteristic is equally due to the high affinity of these cells for the indicator after the color changing induced by the presence of *Z. bailii.* However, the discrimination between those colonies is clear as can be seen in the appended Figure 5.

medium allows the detection of lactic and acetic bacteria as well as yeasts belonging to the non-fermenting flora.

The results are summarized in Table 7.

Table 7 **Number of CFU/ml in 2 contaminated wines, obtained by the method of membrane filtration after 96 hours of incubation in culture medium containing glucose and formic acid (0.4% v/v) at 30°C**

Culture medium	Wine 1	Wine 2
Medium disclosed in Example 1	75 [1]	90 [1] + 170 [2]
WLN	685	620
WLD	10	200

[1] cream-yellowish colored colonies
[2] blue colored colonies, typical of *Z. bailii*

The identification of blue colored colonies and white-yellowish colonies as belonging or not to the *Z. bailii* species was confirmed by molecular methods.

The culture medium described in Example 1 is an ideal culture medium for the isolation of yeasts of the *Zygosaccharomyces bailii* species, allowing the discrimination between this yeast and other yeasts species, just by the color. The WLN and WLD media do not show this differentiation ability that is a characteristic of the medium of the present invention. This property makes this medium superior to those presently commercially available.

Example 6

This example shows the effect of formic acid concentration in the solid culture medium according to the present invention.

A culture medium was prepared as in Example 1, but using different concentrations of formic acid, and inoculation was done with various yeast strains following the same procedure as described in Example 2, as presented in Table 8.

The results obtained are presented in Table 8. For the lower formic acid concentration the basification of the solid culture medium is observed for all the strains belonging to the species *Z. bailii* and *Z. bisporus*. The increase in concentration resulted for 3 *Z. bailii* strains in a slower change in the culture medium color. All the strains of the other tested species induced no color change in the culture medium.

Table 8 **Application of cell suspension drops on the surface of solid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d.
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranaefaciens	13	green	green
Debaryomyces hansenii	2	green	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

The present culture medium is therefor suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, applied as a drop on the surface of the solid medium, for all the tested concentrations of formic acid, after a minimum incubation period of 48 hours. The results obtained show that for 0,3% acid formic concentration, the culture medium according to the invention is appropriate and efficient for the detection of *Z. bailii* and *Z. bisporus* in pure culture suspensions, inoculated in liquid culture medium, after a minimum incubation period of 48 hours. The same is valid for the detection of *Z. bailii* in a medium with 0,5% formic acid concentration. Both concentrations are suitable to guarantee a negative response from the other tested species. However, the medium with 0.5% (v/v) of formic acid is not

the best suited one for the detection of *Z. bailii* strains that show lower tolerance to acid conditions.

Example 7

This Example shows the effect of formic acid concentration in a culture medium according to the present invention.

A culture medium was prepared as in Example 3, but using different concentrations of acid formic and inoculation was done with various yeast strains following the procedure of Example 3, as presented in Table 9.

These results obtained are similar to the ones in Example 6 and are presented in Table 9. In Figure 2 are shown typical responses of yeast strains belonging and not belonging to the *Z. bailii* species.

Table 9 **Inoculation of cell suspensions in liquid medium - response of several yeasts in the culture medium containing glucose and formic acid at different concentrations after 48 hours of incubation at 30°C.**

Species	N. of tested strains	Culture medium color	
		formic acid 0,3% (v/v)	formic acid 0,5% (v/v)
Zygosaccharomyces bailii	12	blue	blue
Zygosaccharomyces bailii	3	blue	blue*
Zygosaccharomyces bisporus	8	blue	n.d.
Zygosaccharomyces florentinus	1	green	green
Saccharomyces bayanus	2	green	green
Saccharomyces cerevisiae	21	green	green
Saccharomyces pastorianus	2	green	green
Pichia membranaefaciens	13	green	green
Debaryomyces hansenii	2	green	green

* the change in the medium color was observed after an additional incubation period of 48-72 hours

n.d. not determined

As in Example 6 the results obtained show that, for 0,3% acid formic concentration, the culture medium according to the present invention is suitable and effective for the detection of *Z. bailii* and *Z. bisporus* from pure culture suspensions, inoculated in liquid media after a minimum incubation

period of 48 hours. The same is valid for the detection of Z. bailii in a medium with 0,5% acid formic concentration.

Example 8

This example shows the effect of formic acid concentration in the culture medium according to the present invention on the medium selectivity.
The procedure of Example 4 was used, but using different concentrations of formic acid in the culture medium.

The results obtained are presented in Table 10. These results and the ones from Example 4 show that the recovery ratio of *Z. bailii* cells in the medium decreases with the increasing of the acid formic concentration, being independent of the presence of other yeast species such those that can be found in contaminated wines. For 2 of these other 3 tested species the recovery ratio also decreases with the increase in the formic acid concentration.

Table 10 **Recovery ratio (%) obtained by the method of membrane filtration after 96 hours of incubation at 30°C.**

Species	*Z. bailii* recovery ratio		
	formic acid 0.2%	formic acid 0.3%	formic acid 0.5%
Zygosaccharomyces bailii	82	78	42
Zygosaccharomyces bailii	82	81	35
Saccharomyces cerevisiae	n.d.	n.d.	n.d.
Zygosaccharomyces bailii	99	94	34
Pichia membranaefaciens	n.d.	n.d.	n.d.
Dekkera anomala	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	30	4	<0.002
Pichia membranaefaciens	55	5.9	<0.004
Dekkera anomala	<0.004	<0.004	<0.004

n.d. not determined

Thus, it was shown that the culture medium according to the present invention has characteristics of a selective and differential culture medium appropriated and highly effective for the detection, identification and

enumeration of *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* yeasts species in samples either containing previously isolated strains of these yeasts or containing mixed yeasts populations. These characteristics of differentiability and selectivity can be optimized. Lower formic acid concentrations provides the medium with remarkable differentiation ability although with lower selectivity. On the other hand, for higher formic acid concentrations the medium is highly selective.

The culture medium can also be supplemented with an inhibitor of bacterial growth, which makes it useful for using with mixed populations samples including also bacteria, as for example food and beverages.

Although the present invention is described based on its preferred embodiments, it should be apparent to any person skilled in the art that variations and modifications within the spirit and scope of the appended claims are possible.

Claims

1. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it comprises a base mineral medium supplemented with vitamins, oligoelements, glucose and formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

2. Culture medium according to claim 1 characterized in that glucose is present in a concentration from 0.05% to 0.1% (p/v), preferably 0.1% (p/v).

3. Culture medium according to claim 1 characterized in that formic acid is present in a concentration, dependent of the desired differentiability and selectivity, from 0.1% to 0.5% (v/v), preferably from 0.2% to 0.4% (v/v).

4. Culture medium according to claim 3 characterized in that the formic acid concentration is preferably 0.4% (v/v).

5. Culture medium according to claim 1 characterized in that the base mineral medium comprises amonium sulphate (0.5% (w/v)), potassium dihydrogen ~~bisulphate~~ phosphate (0.5% (w/v)), magnesium sulphate ~~hetpa~~hepta hydrate (0.05% (w/v)) and calcium chloride dihydrate (0.013% (w/v)); the oligoelements solution A (0.05% (v/v)) comprises boric acid (1.0% (w/v)), potassium iodide (0.2% (w/v)) and sodium molibdate dihydrate (0.4% (w/v)); the oligoelements solution B (0.05% (v/v)) comprises copper sulphate pentahydrate (0.08% (w/v)), iron chloride hexahydrate (0.4% (w/v)), manganese sulphate tetrahydrate (0.8% (w/v)), ~~zinc~~ zinc sulphate heptahydrate (0.8% (w/v)) and hydrochloric acid (HCl 10^{-3}N, 0.8% (v/v)); and the vitamin solution (0.05% (v/v)) comprises biotin (0.001% (w/v)), calcium panthotenate (0.08% (w/v)),

mioinositol (4.0% (w/v)), niacin (0.16% (w/v)), pyridoxine hydrochloride (0.16% (w/v)) and thiamin hydrochloride (0.16% (w/v)).

6. Culture medium according to claim 1 characterized in that the acid-base indicator is one having a pK_i between 4.5 and 4.8, preferably bromocresol green.

7. Culture medium according to claim 6 characterized in that the pH is adjusted to 4.3-4.8, preferably 4.5.

8. Culture medium according to claim 1 characterized in that it further contains an antibiotic inhibitor of bacterial growth, in the usually used concentrations for this purpose, for use with mixed population samples containing bacteria.

9. A culture medium according to any previously claim characterized in that it contains all the ingredients except agar, that is in its liquid form.

10. A differential and selective culture medium for *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts, characterized in that it is composed of

Glucose	0.1% (w/v)
Formic acid	0.4% (v/v)
Base Medium:	
Ammonium sulphate	0.5% (w/v)
Potassium dihydrogen ~~sulphate~~ phosphate	0.5% (w/v)
Magnesium sulphate ~~hetpa~~hepta hydrate	0.05% (w/v)
Calcium chloride dihydrate	0.013% (w/v)
Bromocresol green	0.005% (w/v)
Agar	2.0% (w/v)
Oligoelements Solution A	0.05% (v/v)
Boric acid	1.0% (w/v)

	Potassium Iodide	0.2% (w/v)
	Sodium molibdate dihydrate	0.4% (w/v)
Oligoelements Solution B		0.05% (v/v)
	Copper sulphate pentahydrate	0.08% (w/v)
	Iron chloride hexahydrate	0.4% (w/v)
	Manganese sulphate tetrahydrate	0.8% (w/v)
	Zinc sulphate heptahydrate	0.8% (w/v)
	Hydrochloric acid, HCl 10^{-3}N,	0.8% (v/v)
Vitamin Solution		0.05% (v/v)
	Biotin	0.001% (w/v)
	Calcium panthotenate	0.08% (w/v)
	Mioinositol	4.0% (w/v)
	Niacin	0.16% (w/v)
	Pyridoxine hydrochloride	0.16% (w/v)
	Thiamin hydrochloride	0.16% (w/v)

the pH being adjusted to pH 4.5 with HCl 1M.

11. Culture medium according to any previously claim characterized in that the medium is prepared by dissolving the base medium compounds in 4/5 of the estimated deionized water volume, the sterilization being accomplished in autoclave at 121°C, for 20 minutes, by dissolving the other medium compounds in the remaining water so that the final concentration of these compounds equals the desired values, the sterilization being accomplished by filtration, annealing this solution and the base medium at about 50 ± 5°C, before mixing the same and to adjust the final pH value to the desired value.

12. Process for the detection of *Zygosaccharomyces bailii* e *Zygosaccharomyces bisporus* yeasts characterized by the use of a differential and selective culture medium for the referred yeast species, comprising a base mineral medium supplemented with vitamins, oligoelements, glucose and

formic acid as the only carbon and energy sources, an appropriated acid-base indicator and, optionally an antibiotic inhibitor of bacterial growth and agar.

13. Process according to claim 12, characterized in that the acid-base indicator is bromocresol green and in that, after inoculating the referred culture medium with a sample containing *Zygosaccharomyces bailii* and/or *Zygosaccharomyces bisporus* yeasts and incubating in conditions appropriated for the growth of the referred yeasts, it is possible to conclude for the presence of said yeasts species, by means of a medium color change from green to blue after about 48 hours, and if desired to number said yeasts species, by development of blue colored colonies after about 96 hours.

14. Process according to claims 12 and 13 characterized in that it is applied to the detection and numbering yeasts of the *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* species in wines, as well as in other beverages or food containing or not mixed yeast populations.

15. Use of a culture medium according to claims 1 to 11, to be included in yeast identification galleries.

16. Use of a culture medium according to claims 1 to 11 in an industry, particularly in the quality and process control in the food and beverage industry.

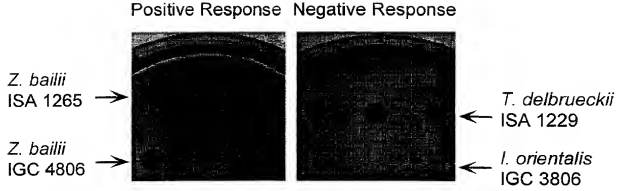

Fig. 1

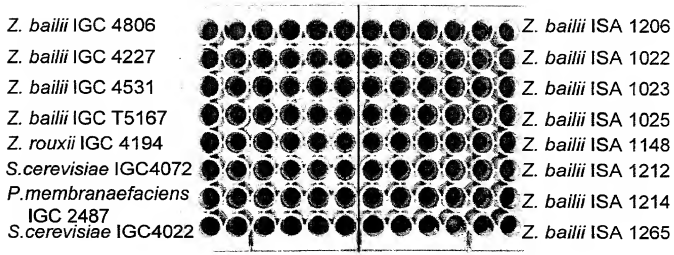

Fig. 2

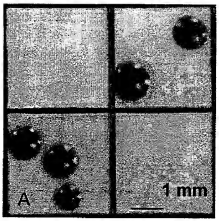

Fig. 3

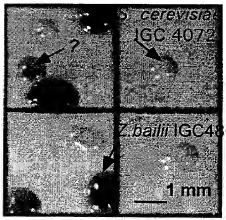

Fig. 4

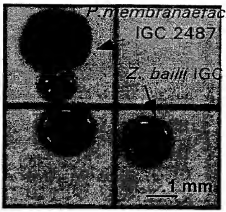

Fig. 5

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Declaration, Power of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CULTURE MEDIUM FOR DETECTION OF ZYGOSACCHAROMYCES

the specification of which

☐ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____.

☒ was filed as PCT international application

Number PCT/PT00/00004

on May 31, 2000,

and was amended under PCT Article 19

on November 08, 2000 (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
102305	Portugal	31 May 1999	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

?????	?????
(Application Number)	(Filing Date)
?????	?????
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/PT00/00004	31 May 2000	?????
?????	?????	?????
?????	?????	?????

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And we (I) hereby appoint the following registered practitioner(s):

as our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to

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We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Cecilia LEÃO
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~~?????~~ October 28, 2001
Signature of Inventor

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Date

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Citizen of: Portugal
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Signature of Inventor

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Date

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NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

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Date

Residence: ~~?????~~

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~~?????~~

Citizen of: ~~?????~~

Mailing Address: ~~?????~~

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~~?????~~

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

~~?????~~

Date

Residence: ~~?????~~

~~?????~~

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Citizen of: ~~?????~~

Mailing Address: ~~?????~~

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